RhoA Silencing Reverts the Resistance to Doxorubicin in Human Colon Cancer Cells

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Abstract
The efficacy of doxorubicin in the treatment of cancer is limited by its side effects and by the onset of drug resistance. Reverting such resistance could allow the decrease of the dose necessary to eradicate the tumor, thus diminishing the toxicity of the drug. We transfected doxorubicin-sensitive (HT29) and doxorubicin-resistant (HT29-dx) human colon cancer cells with RhoA small interfering RNA. The subsequent decrease of RhoA protein was associated with the increased sensitivity to doxorubicin in HT29 cells and the complete reversion of doxorubicin resistance in HT29-dx cells. RhoA silencing increased the activation of the nuclear factor-κ-B pathway, inducing the transcription and the activity of nitric oxide synthase. This led to the tyrosine nitration of the multidrug resistance protein 3 transporter (MRP3) and contributed to a reduced doxorubicin efflux. Moreover, RhoA silencing decreased the ATPase activity of P-glycoprotein (Pgp) in HT29 and HT29-dx cells as a consequence of the reduced expression of Pgp. RhoA silencing, by acting as an upstream controller of both MRP3 nitration and Pgp expression, was effective to revert the toxicity and accumulation of doxorubicin in both HT29 and HT29-dx cells. Therefore, we suggest that inactivating RhoA has potential clinical applications and might in the future become part of a gene therapy protocol.

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Introduction
Resistance to anticancer drugs, including doxorubicin, is still a major cause in the failure of chemotherapy in cancer patients (1, 2). The mechanisms involved in drug resistance are complex and multifactorial and may depend on an inadequate drug exposure and/or to genetic and epigenetic alterations in the cancer cell itself (3). Moreover, doxorubicin efficacy is limited because of cardiotoxicity, a dose-dependent phenomenon and an important adverse effect that impairs the patients’ outcome and survival (4).

Small Rho GTPases (RhoA, Rac, and Cdc42) play a key role in the regulation of tumor growth, migration, and response to therapy (5). They are localized at cell membrane and become activated with the stimulation of cell surface receptors. In their GTP-bound active state, Rho proteins bind in turn to effector proteins (5). The expression of Rho GTPases frequently increases with malignancy (5). Thus, the modulation of these Rho-driven mechanisms may influence therapeutic efficacy and/or side effects of conventional antineoplastic therapy. We have previously shown that statins can revert doxorubicin resistance in malignant mesothelioma cells (6). Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, the enzyme catalyzing the rate-limiting step in cholesterol synthesis. Therefore, statins cause a dramatic decrease of both cholesterol and isoprenoid intermediates and impair the isoprenylation and activity of different enzymes, such as the small Rho GTPases (7). We have previously shown that statins can revert doxorubicin resistance in HT29 cells and Doxorubicin-Resistant (HT29-dx) Colon Cancer Cells.

Results
Specific RhoA Silencing in Human Doxorubicin-Sensitive (HT29) and Doxorubicin-Resistant (HT29-dx) Colon Cancer Cells
To investigate the involvement of RhoA in doxorubicin-induced cytotoxicity, HT29 and HT29-dx cells were transfected
HT29 RhoA-KD and HT29-dx RhoA-KD Cells Accumulate More Doxorubicin and Are More Sensitive to Its Cytotoxic Effects than Control HT29 and HT29-dx Cells

After a 6-h incubation with doxorubicin, drug accumulation was significantly higher in HT29 RhoA-KD than in HT29 cells (Fig. 2). As we previously described (9), the accumulation of doxorubicin in HT29-dx cells was ~50% of that measured in HT29 cells (Fig. 2). When HT29-dx cells were silenced with RhoA siRNA, the intracellular amount of doxorubicin was the same as in the nonsilenced HT29 cells (Fig. 2). Under the same experimental conditions, we measured also the cytotoxic effects of the drug through the release of LDH activity in the extracellular medium. The LDH release was significantly lower in HT29-dx cells compared with HT29 cells and significantly increased in both RhoA-silenced cell types versus the respective nonsilenced control cells (Fig. 2). RhoA silencing did not exert a significant increase of LDH release in the extracellular medium in the absence of doxorubicin (extracellular LDH versus total LDH: HT29 = 21.93 ± 3.82%, HT29 RhoA-KD = 20.25 ± 2.19%, HT29-dx = 22.55 ± 3.60%, and HT29-dx RhoA-KD = 20.42 ± 3.57%; n = 3). These results suggest that HT29 and HT29-dx cells transfected with RhoA siRNA accumulate greater amounts of doxorubicin and are more sensitive to its cytotoxic effects.

RhoA Silencing Increases Nuclear Factor-κB Translocation into the Nucleus of HT29 Cells

A relationship between Rho kinase and IκB kinase (IKK) α/β/IκBα/nuclear factor-κB (NF-κB) pathway has been hypothesized in other cell types (6, 10, 11). Therefore, we investigated whether RhoA inhibition in HT29 cells may lead to an IKK-mediated nuclear translocation of NF-κB. As shown in Fig. 3A, RhoA silencing induced nuclear translocation of NF-κB in both with specific RhoA small interfering RNA (siRNA) to obtain a knockdown (KD) phenotype. Silenced cells were designed as HT29 RhoA-KD and HT29-dx RhoA-KD cells. As shown by Western blot analysis, after 72 h from the transfection with 50 pmol of the RhoA siRNA, HT29 RhoA-KD and HT29-dx RhoA-KD cells expressed very low level of RhoA when compared with parental cells (78 ± 6% of decrease in HT29 RhoA-KD cells versus HT29 cells, n = 3, P < 0.001, and 82 ± 7% of decrease in HT29-dx RhoA-KD cells versus HT29-dx cells, n = 3, P < 0.001), whereas the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the product of a housekeeping gene unrelated to RhoA protein, was unchanged (Fig. 1). Moreover, when we incubated cells with a nontargeting 20- to 25-nucleotide siRNA, there was no change in the expression of both RhoA and GAPDH. To control the specificity of the transfection even further, we transfected cells with 50 pmol of the GAPDH siRNA. Western blot analysis showed that HT29 GAPDH-KD and HT29-dx GAPDH-KD cells expressed very low level of GAPDH when compared with parental cells and negative control cells, whereas RhoA expression was unchanged throughout the different experimental conditions (Fig. 1). Cell viability was not affected by transfection, as lactate dehydrogenase (LDH) release in the supernatant after 24, 48, and 72 h from the transfection was the same as in nontransfected cells (data not shown).
cell types, whereas nonsilenced HT29 and HT29-dx cells showed undetectable levels of NF-κB in the nucleus. Western blot analysis on nuclear extracts confirmed the electrophoretic mobility shift assay results: the p50/p65 dimer of NF-κB was detectable only in the nuclei of HT29 RhoA-KD and HT29-dx RhoA-KD cells (Fig. 3B). Under basal conditions, members of the inhibitory IκB family proteins bind directly to the NF-κB dimer in the cytoplasm, preventing its nuclear localization (12). When IκBα is phosphorylated by IKK complex, ubiquitinated, and degraded by the proteasome, NF-κB is free to translocate and bind to DNA on target genes (13-15). Indeed, we observed that RhoA silencing markedly decreased the level of the NF-κB inhibitor IκBα and increased the phosphorylation/activation of IκBα and IKKα in HT29 RhoA-KD and HT29-dx RhoA-KD cells versus their respective controls (Fig. 3C). The onset of phospho-IκBα in RhoA-silenced cells suggests that the decrease of IκBα is likely to be caused by increased proteasome-mediated degradation of the phosphorylated protein. RhoA silencing caused the onset of detectable IKK activity, as inferred by the ability of RhoA-KD cell lysates to phosphorylate in vitro the substrate IκBα (Fig. 3D) from the cell lysate of SV40-positive mesothelioma cells, in the presence of the proteasome inhibitor MG132 (to avoid the immediate destruction of phospho-IκBα by the proteasome possibly still present in the cell lysate). MG132 prevented the disappearance of IκBα, the total amount of which remained constant throughout the assay, despite its phosphorylation (Fig. 3D). The RhoA kinase inhibitor Y27632 (10 μmol/L, 1 h) significantly decreased the activity of RhoA kinase (data not shown) and enhanced the phosphorylation and kinase activity of IKK in both HT29 and HT29-dx cells (Fig. 4). The effect of Y27632 was comparable with the one induced by RhoA silencing, and no additive effects were observed when Y27632 and RhoA siRNA were coincubated with the cells. Neither Y27632 nor RhoA silencing affected the total amount of IKK (Fig. 4).

RhoA Silencing Increases the NOS Activity

We previously observed that the resistance to doxorubicin could be reverted by incubating HT29-dx cells with inducers of nitric oxide (NO) synthesis (cytokines and atorvastatin; ref. 9). Therefore, we investigated whether the enhanced sensitivity to doxorubicin in HT29 RhoA-KD cells and the reversion of doxorubicin resistance in HT29-dx RhoA-KD cells versus their respective controls (Fig. 3C). The onset of phospho-IκBα in RhoA-silenced cells suggests that the decrease of IκBα is likely to be caused by increased proteasome-mediated degradation of the phosphorylated protein. RhoA silencing caused the onset of detectable IKK activity, as inferred by the ability of RhoA-KD cell lysates to phosphorylate in vitro the substrate IκBα (Fig. 3D) from the cell lysate of SV40-positive mesothelioma cells, in the presence of the proteasome inhibitor MG132 (to avoid the immediate destruction of phospho-IκBα by the proteasome possibly still present in the cell lysate). MG132 prevented the disappearance of IκBα, the total amount of which remained constant throughout the assay, despite its phosphorylation (Fig. 3D). The RhoA kinase inhibitor Y27632 (10 μmol/L, 1 h) significantly decreased the activity of RhoA kinase (data not shown) and enhanced the phosphorylation and kinase activity of IKK in both HT29 and HT29-dx cells (Fig. 4). The effect of Y27632 was comparable with the one induced by RhoA silencing, and no additive effects were observed when Y27632 and RhoA siRNA were coincubated with the cells. Neither Y27632 nor RhoA silencing affected the total amount of IKK (Fig. 4).
shown in Fig. 5A, the p65 binding to iNOS promoter/enhancer region was detectable in both RhoA-KD cell types, but not in nonsilenced HT29 and HT29-dx cells. iNOS mRNA was low in nonsilenced HT29 and HT29-dx cells, whereas it was significantly increased in both RhoA-KD cell populations (Fig. 5B). Furthermore, NOS activity, which was lower in HT29-dx cells than in HT29 cells, as previously described (9), was significantly enhanced by RhoA silencing in both cell types (Fig. 5C).

RhoA Silencing Increases the Sensitivity to Doxorubicin in HT29 RhoA-KD and HT29-dx RhoA-KD Cells via NF-κB and NOS Activation

The increase in the level and cytotoxic effects of doxorubicin in RhoA-silenced cells was mediated by an increase in NO synthesis, driven by the NF-κB translocation into the nucleus. Indeed, in the presence of packed human erythrocytes (a reservoir of the potent NO scavenger oxyhemoglobin), of L-arginine (a NOS inhibitor), or of SN50 (a specific NF-κB nuclear translocation inhibitor), RhoA silencing did not result in increasing drug levels and LDH release in HT29 and HT29-dx cells after incubation with doxorubicin (Fig. 6).

RhoA Silencing Induces Tyrosine Nitration in the MDR Protein 3 Transporter

We previously observed that NO could elicit increased drug sensitivity by nitrating tyrosine residues in the drug efflux pump MDR protein 3 transporter (MRP3; ref. 9). Therefore, we investigated whether the enhanced NOS activity observed in RhoA-silenced cells was accompanied by increased nitrations of MRP3 tyrosine residues. As we previously observed (9), MRP3 expression was very low in doxorubicin-sensitive HT29 cells and increased remarkably in doxorubicin-resistant HT29-dx cells (Fig. 7A and B). In both HT29 and HT29-dx cells, RhoA silencing induced tyrosine nitration (otherwise not detectable) in the MRP3 transporter. This nitration was undetectable when silenced cells were coincubated with RBCs, L-NMMA, and SN50 (Fig. 7A and B). No nitration was detected on the P-glycoprotein (Pgp; data not shown), another drug membrane transporter involved in MDR (16, 17), confirming our previous observations in HT29 and HT29-dx cells (9).

The nitration of MRP3 was detectable to the pump activity: indeed, RhoA siRNA induced a significant tyrosine nitration of MRP3 and decreased the $V_{\text{max}}$ of doxorubicin efflux in both HT29 and HT29-dx cells (Fig. 7C). In HT29-dx–silkced cells, the $V_{\text{max}}$ was superimposable to the one observed in nonsilenced HT29 cells. On the contrary, in silenced cells incubated with RBCs, L-NMMA, and SN50, the tyrosine nitration of MRP3 was abrogated and the $V_{\text{max}}$ of drug efflux was the same as in nonsilenced cells (Fig. 7C).

RhoA siRNA Reduces the ATPase Activity of Both Pgp and MRP3

We measured the ATPase activity of Pgp and MRP3, isolated from silenced and nonsilenced HT29 and HT29-dx cells (Fig. 8). Both Pgp and MRP3 exhibited increased ATPase activity in HT29-dx cells when compared with HT29 cells. RhoA silencing decreased the ATPase activity of both proteins in each cell population. In HT29-dx cells, richer in Pgp and MRP3, the reduction of ATPase activity caused by RhoA siRNA was greater: indeed, the enzyme activity decreased to the same level observed in non silenced HT29 cells (Fig. 8).

RhoA Silencing Causes Also a Decrease of the Pgp Expression, Which Contributes to the Doxorubicin Resistance

Because RhoA silencing decreased the activity of both Pgp and MRP3 but caused the nitrations of MRP3 only, we investigated whether RhoA siRNA might affect the expression of Pgp (Fig. 9). The Pgp mRNA level was greater in HT29-dx cells than in HT29 cells: RhoA siRNA significantly decreased the amount of Pgp mRNA in both cell populations (Fig. 9A). This effect was likely mediated by the activation of NF-κB; indeed, in the presence of the NF-κB inhibitor SN50, the Pgp mRNA remained high in RhoA-silenced HT29 and HT29-dx cells (Fig. 9A).

HT29-dx cells exhibited a greater amount of Pgp, as assessed by Western blotting (Fig. 9B and C) and immunofluorescence analysis (Fig. 9D and E). RhoA silencing significantly decreased the amount of Pgp in both HT29 and HT29-dx cells (Fig. 9B–E).

To investigate whether the doxorubicin resistance was significantly affected by the Pgp expression, we both overexpressed and silenced Pgp in HT29 and HT29-dx cells and then...
HT29-dx.

**Materials and Methods.**

Measurements were done in duplicate. Columns, mean (n = 3); bars, SE. *P < 0.05 versus HT29; **P < 0.05 versus HT29-dx.

**Discussion.**

Chemotherapy has improved remission and survival in many solid and hematologic malignancies. The efficacy of chemotherapy, however, may be hindered by innate or acquired cross-resistance to different chemotherapeutic agents, such as the anthracyclines. MDR is regarded as a multifactorial process and may alter the prognosis of some types of solid cancers, such as colon, lung, and breast, and certain types of adult leukemia (1). Several studies have indicated that a cardinal feature of MDR is the greater rate of extrusion of antineoplastic drugs (1). ATP-binding cassette (ABC) transporters recognize many antineoplastic agents as their substrates. The discovery of ABC transporters has led to investigate whether MDR can be reverted (2, 19). Indeed, understanding the pathways involved in this resistance may lead to new ways to treat cancers.

In the present study, we hypothesized that a common mechanism may both enhance the efficacy of doxorubicin in sensitive cancer cells and correct the resistance to the drug in resistant cancer cells. With this target in mind, we used the human colon cancer cells HT29. These cells express low basal levels of Pgp and MRP3 and therefore are a good model of...
doxorubicin-sensitive cells (9). HT29 cells easily convert into doxorubicin-resistant cells (HT29-dx), which express higher levels of Pgp and MRP3 compared with HT29 cells, when they become exposed to constant low doses of the drug (9). In human mesothelioma cells, which similarly overexpress both Pgp and MRP3 and are constitutively resistant to doxorubicin, we have previously corrected the resistance to doxorubicin by inducing NF-κB activation and NO synthesis with statins (6); we have found that statins lower the amount of active RhoA and the level of Rho-associated kinase activity. Indeed, both the Rho kinase inhibitor Y27632 and the RhoA inhibitor toxin B mimic the effects of the statins and are able to induce NF-κB and NO synthesis and to revert the resistance to doxorubicin (6).

We then asked the question whether the efficacy of doxorubicin could be enhanced by the specific silencing of RhoA. We have now observed that the RhoA-targeted RNA interference technique causes a decrease of RhoA protein expression, increases the sensitivity to doxorubicin in drug-sensitive HT29 cells, and completely reverts the resistance to doxorubicin in HT29-dx cells in a NF-κB–dependent and iNOS-dependent way.

The NF-κB family contains a large number of dimeric transcription factors, such as the most studied p50/p65 dimer, and controls several genes involved in the response to cellular stress, cell survival, and proliferation (20). The inhibitor protein IκBα sequesters NF-κB in the cytoplasm of resting cells. Different stimuli, such as inflammatory cytokines, bacterial lipopolysaccharide, or oxidative stress, promote IκBα inactivation through phosphorylation, ubiquitylation, and proteasomal degradation, thus allowing the nuclear translocation of NF-κB (20). IKK complex is responsible for the phosphorylation of IκBα on Ser32 and Ser36. The IKKα subunit becomes fully active when phosphorylated on Ser176 and Ser180 (20).

We observed that HT29 and HT29-dx cells did not exhibit any basal NF-κB activation, a feature similar to other resting nontransformed cells. Specifically, NF-κB binding on DNA was absent, IκBα was abundant, whereas IKKα was neither phosphorylated nor active as a kinase. It has been reported that statins may induce or prevent NF-κB activity (10, 11, 21, 22) depending on the cell type, the different experimental conditions, the basal level of NF-κB activity, and the prevalent isoforms of NF-κB dimer in each cell line. In HT29 and HT29-dx cells, RhoA silencing elicited a clear translocation of the p50/p65 NF-κB dimer into the nucleus, reduced the level of IκBα protein, and enhanced IKKα phosphorylation and kinase activity. It has been suggested that the inhibition of RhoA kinase may induce IKK activity, allowing the p65 binding to target genes (23, 24). In the present work, both RhoA silencing and RhoA kinase inhibition activated IKK complex to the same extent. It is conceivable that the inhibition of RhoA kinase is responsible for IKK activation and NF-κB translocation in HT29 and HT29-dx cells.

All the agents that activate the NF-κB pathway enhance the expression of iNOS and the production of NO (10, 21, 25, 26). Our study was focused on the p50/p65 NF-κB dimer, which is a direct activator of iNOS gene (23). The chromatin immunoprecipitation assay and real time-PCR experiments confirmed that p65 is implicated in the iNOS transcription in RhoA-silenced HT29 and HT29-dx cells. Besides controlling fundamental cellular functions, such as growth, differentiation, and apoptosis (20), NO may modulate the activity of different enzymes and membrane proteins through cysteine S-nitrosylation and/or tyrosine nitration (27). We have previously shown that Pgp and MRP3, two ABC membrane transporters, which actively extrude several antineoplastic drugs, may be target of NO: as a consequence of tyrosine nitration, the efflux of doxorubicin is inhibited (9). In human malignant mesothelioma cells, which overexpress both Pgp and MRP3, mevastatin and simvastatin significantly induce NO synthesis, elicit Pgp nitration, and decrease the resistance to doxorubicin (6). In HT29 and HT29-dx cells, RhoA siRNA significantly increased NO synthesis, elicit Pgp nitration, and consequence of tyrosine nitration, the efflux of doxorubicin is inhibited (9). In human malignant mesothelioma cells, which overexpress both Pgp and MRP3, mevastatin and simvastatin significantly induce NO synthesis, elicit Pgp nitration, and decrease the resistance to doxorubicin (6). In HT29 and HT29-dx cells, RhoA silencing elicited a clear translocation of the p50/p65 NF-κB dimer into the nucleus, reduced the level of IκBα protein, and enhanced IKKα phosphorylation and kinase activity. It has been suggested that the inhibition of RhoA kinase may induce IKK activity, allowing the p65 binding to target genes (23, 24). In the present work, both RhoA silencing and RhoA kinase inhibition activated IKK complex to the same extent. It is conceivable that the inhibition of RhoA kinase is responsible for IKK activation and NF-κB translocation in HT29 and HT29-dx cells.

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On the contrary, when either NO was scavenged or NOS activity and expression were prevented, no nitration on MRP3 was detected and the $V_{\text{max}}$ of the drug efflux did not change even in the presence of RhoA siRNA. No nitration was detected on Pgp probably as a consequence of the low number of accessible tyrosines on the Pgp colon isoform (9).

However, RhoA silencing significantly decreased the ATPase activity of both MRP3 and Pgp. Therefore, we investigated whether RhoA siRNA decreased the expression of Pgp: our results showed indeed that RhoA silencing lowered both Pgp mRNA and protein in HT29 and HT29-dx cells. When RhoA-silenced cells were incubated with the Pgp inhibitor verapamil, no further effect on intracellular doxorubicin accumulation was observed, an indirect evidence that Pgp protein is absent or strongly inhibited in RhoA-silenced cells (data not shown).

Several concerns have also been raised about the link between NF-$\kappa$B activation and Pgp expression. Some experimental works suggest that, when NF-$\kappa$B is constitutively high, cells escape more easily apoptosis induced by chemotherapeutic drugs (20, 28). In other models, NF-$\kappa$B is induced by anticancer drugs, which in parallel enhance the expression of Pgp (29): in these works, the decrease of NF-$\kappa$B activation has been associated with reduced expression of Pgp (30). On the contrary, other works reported that the activation of NF-$\kappa$B is necessary for anticancer drugs to induce apoptosis and it represses the Pgp promoter activity (31, 32). It is generally acknowledged that the role of NF-$\kappa$B in chemoresistance is highly cell specific depending on the basal level of NF-$\kappa$B, the drug investigated, and the way of drug exposure (30). In our experimental conditions, NF-$\kappa$B was undetectable and Pgp was constitutively expressed in both HT29 and HT29-dx cells, although to different extents; the activation of NF-$\kappa$B by RhoA silencing decreased the Pgp mRNA, which was restored by preventing NF-$\kappa$B activation in silenced cells. Interestingly, our results showed that RhoA silencing decreased the ATPase activity of both Pgp and MRP3, albeit via different molecular mechanisms: reduced expression of Pgp and tyrosine nitration of MRP3.

When transfected with the Pgp gene, HT29 and HT29-dx cells accumulated significantly less doxorubicin: this effect was prevented when Pgp was silenced. In Pgp-silenced cells, the drug accumulation was about half of the one observed in RhoA-silenced cells, whereas double (Pgp + RhoA)-silenced
cells accumulated as much doxorubicin as the one contained in RhoA-silenced cells. These results suggest that RhoA acts as an upstream controller of Pgp expression. In summary, our data suggest that silencing of RhoA induces the nuclear translocation of the transcription factor NF-κB and increases consequently the NOS activity. NO in turn nitrates the doxorubicin efflux from cells and enhancing the cytotoxic effect of the drug (Fig. 1). Because a large number of cellular functions are controlled by small G proteins, many efforts have been made to modulate the prenylation and the activation of NF-κB represses the Pgp transcription, further reducing the doxorubicin efflux from cells and enhancing the cytotoxic effect of the drug (Fig. 1). Because a large number of cellular functions are controlled by small G proteins, many efforts have been made to modulate the prenylation and the activity of Rho in cancer cells (5). Statins have already been related to the reversal of doxorubicin resistance in MDR human cancer cell lines (6, 33). Nevertheless, the direct targeting of RhoA by siRNA seems to be a more specific tool than the less specific inhibition of 3-hydroxy-3-methylglutaryl CoA reductase with statins. Recently, modulation of MDR by transient and stable expression of MDR1-targeted siRNA has been reported (34-36). In addition, RhoA silencing has been successfully applied as an anticancer tool in vivo: in murine xenografts of human metastatic breast cancer, RhoA siRNA reduced the proliferation and migration of tumor cells (37, 38). Our study shows that specific silencing of RhoA may improve doxorubicin efficacy in colon cancer in vitro, allowing the reduction of the overall dose of doxorubicin, the increase of the therapeutic benefits/adverse effect ratio, and the reversal of the resistance, which is often rapidly acquired during the chemotherapy.

Materials and Methods

Materials

Fetal bovine serum (FBS) and RPMI 1640 were supplied by BioWhittaker; plasticware for cell culture was from Falcon (Becton Dickinson). Electrophoresis reagents were obtained from Bio-Rad Laboratories; the protein content of cell monolayers and cell lysates was assessed with the bicinchoninic acid kit from Sigma Chemical Co. When not otherwise specified, all the other reagents were purchased from Sigma Chemical.

Cells

Human colon cancer cells (HT29 cell line) were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% l-glutamine and maintained in a humidified atmosphere at 37°C and 5% CO2. A subpopulation of HT29 cells, after 20 to 25 passages in RPMI 1640 + 10% FBS supplemented with 68 nmol/L doxorubicin, exhibited a significantly lower intracellular level of drug after a doxorubicin bolus (5 μmol/L) and, at the same time, became more resistant to the toxic effects of the drug (see Results); these cells were named HT29-dx and subsequently cultured in RPMI 1640 containing 34 nmol/L doxorubicin ("maintenance dose"); ref. 9).

RhoA siRNA Transfection

Cells were plated in 35-mm-diameter Petri dishes (150,000 per dish) and cultured in RPMI 1640 containing 10% FBS. After 24 h, cells were washed once with 2 mL siRNA transfection medium (Santa Cruz Biotechnology) and incubated for 6 h with 1 mL siRNA transfection medium containing 5 μL of siRNA transfection reagent (Santa Cruz Biotechnology). In each set of experiments, one dish was treated with 50 pmol of Control siRNA-A (Santa Cruz Biotechnology), a nontargeting 20- to 25-nucleotide siRNA designed as a negative control, instead of RhoA siRNA. At the end of the incubation, 1 mL of RPMI 1640 containing 1% penicillin/streptomycin and 20% FBS was added for 24 h. Subsequently, cells were washed and cultured for 72 h in RPMI 1640 with 1% penicillin/streptomycin and 10% FBS. Cellular toxicity was assessed by measuring the extracellular LDH release (see below) after 24, 48, and 72 h from transfection. To verify the siRNA efficacy, cells were lysed and the expression of RhoA protein was analyzed by Western blotting using an anti-RhoA antibody (diluted 1:250 in PBS-1% bovine serum albumin (BSA); Santa Cruz Biotechnology). To assess the siRNA specificity for RhoA, we checked the expression of GAPDH, a product of a housekeeping gene in both transfected and untransfected cells. An analogous protocol of target gene silencing was followed to knock down Pgp (Santa Cruz Biotechnology). To assess the effectiveness of siRNA, we checked the expression of Pgp by Western blot analysis, as reported below.

Electrophoretic Mobility Shift Assay

Cells were plated in 60-mm-diameter dishes at confluence and all procedures for nuclear protein extraction were done at 4°C using ice-cold reagents as described (39). The probe containing the NF-κB oligonucleotide consensus sequence was labeled with [γ-32P]ATP (3,000 Ci/mmol, 250 μCi; Amersham International) using T4 polynucleotide kinase (Roche). The sequence of oligonucleotide was (binding site underlined) 5'-AGTTGAGGGGACTTCCAGG-3' (Promega Corp.).
Extracts (10 μg) were incubated for 20 min with 20,000 cpm of 32P-labeled double-stranded oligonucleotide at 4°C. In super-shift assay, nuclear extracts were preincubated for 30 min at room temperature with 2 μL of the anti-p50 antibody (Santa Cruz Biotechnology) or anti-p65 antibody; then, the reaction mixture containing the 32P-labeled double-stranded oligonucleotide was added and sample was treated as described previously. The DNA-protein complex was separated on a nondenaturing 4% polyacrylamide gel in TBE buffer [0.4 mol/L Tris, 0.45 mol/L boric acid, 0.5 mol/L EDTA (pH 8.0)]. After electrophoresis, the gel was dried and autoradiographed by exposure to X-ray film for 48 h.

**Western Blot Analysis**

Cells were directly solubilized in the lysis buffer (25 mM HEPES, 135 mM NaCl, 1% NP40, 5 mM EDTA, 4% polyacrylamide gel in TBE buffer [0.4 mol/L Tris, 0.45 mol/L boric acid, 0.5 mol/L EDTA (pH 8.0)]. After electrophoresis, the gel was dried and autoradiographed by exposure to X-ray film for 48 h.

![Western Blot](image)

**FIGURE 9.** Effects of RhoA silencing on drug efflux pump Pgp gene and protein expression in HT29 and HT29-dx cells. **A.** HT29, RhoA-silenced HT29 (siRNA), HT29-dx, and RhoA-silenced HT29-dx (siRNA) cells were incubated for 5 h with SN50 (SN; 25 μmol/L), a specific inhibitor of NF-κB nuclear translocation. Pgp mRNA expression was measured as described (see Materials and Methods). Measurements were done in duplicate. Columns, mean (n = 3); bars, SE. *, P < 0.01 versus HT29; †, P < 0.001 versus HT29-dx; ‡, P < 0.001 versus siRNA. **B.** Western blot detection of Pgp protein in HT29, RhoA-silenced HT29 (HT29 siRNA), HT29-dx, and RhoA-silenced HT29-dx (HT29-dx siRNA) cells. Whole-cell lysates were analyzed by Western blotting with anti-Pgp antibody. An anti-GAPDH antibody was used as a control of equal protein loading (see Materials and Methods for details). The figure is representative of three experiments with similar results.

**C.** Relative band intensities, calculated from the results represented in B using ImageJ software (http://rsb.info.nih.gov/ij/) and expressed as arbitrary units, are presented as mean ± SE (n = 3). *, P < 0.05 versus HT29; †, P < 0.05 versus HT29-dx.

**D.** Micrographs representative of immunofluorescence staining for Pgp in nonpermeabilized HT29, RhoA-silenced HT29 (HT29 siRNA), HT29-dx, and RhoA-silenced HT29-dx (HT29-dx siRNA) cells. Nonpermeabilized cells were analyzed by immunofluorescence with anti-Pgp antibody and a nonimmune isotypic antibody was used as a control (see Materials and Methods for details). The figure is representative of three experiments with similar results.

**E.** Semiquantitative analysis of Pgp expression as detected by immunofluorescence staining (see examples in D) in nonpermeabilized HT29, RhoA-silenced HT29 (HT29 siRNA), HT29-dx, and RhoA-silenced HT29-dx (HT29-dx siRNA) cells. Results are expressed as % versus HT29 cells (n = 3). *, P < 0.05 versus HT29; †, P < 0.05 versus HT29-dx.
1 mmol/L EGTA, 1 mmol/L ZnCl₂, 50 mmol/L NaF, 10% glycerol) supplemented with protease inhibitor cocktail set III (100 mmol/L 4-(2-aminoethyl)benzenesulfonylfluoride, 80 μmol/L aprotinin, 5 mmol/L bestatin, 1.5 mmol/L E-64, 2 mmol/L leupeptin, 1 mmol/L pepstatin; Calbiochem), 2 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate. Whole-cell extracts containing 30 μg of proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane sheets (Immobilon-P, Millipore), and probed with the following antibodies: anti-IκBα (from rabbit, diluted 1:500 in PBS-1% BSA), anti-phospho(Ser32)-IκBα antibody (from mouse, diluted 1:250 in PBS-1% BSA), anti-IKKα (from rabbit, diluted 1:500 in PBS-1% BSA), anti-phospho(Ser180)-IKKα (from rabbit, diluted 1:250 in PBS-1% BSA; Cell Signaling Technology, Inc.), anti-GAPDH (from rabbit, diluted 1:500 in PBS-1% BSA), anti-RhoA (from rabbit, diluted 1:500 in PBS-1% BSA), and anti-Pgp (from rabbit, diluted 1:250 in PBS-1% BSA). Nuclear proteins (30 μg) obtained as described (39) were separated by SDS-PAGE, transferred on PVDF membrane, and probed with anti-p50 (from mouse, diluted 1:250 in PBS-1% BSA) and anti-p65 (from rabbit, diluted 1:500 in PBS-1% BSA) antibodies.

To measure MRP3 expression, MRP3 protein was immunoprecipitated overnight from the whole-cell lysates with the goat polyclonal anti-MRP3 antibody (diluted 1:250; Santa Cruz Biotechnology), separated by SDS-PAGE, transferred to PVDF membrane sheets, and probed with the same antibody. To assess the presence of nitrated MRP3, the whole cellular extract was

**FIGURE 10.** Effect of Pgp and RhoA silencing on doxorubicin resistance in HT29 and HT29-dx cells. A. Doxorubicin accumulation in HT29 (white columns) and HT29-dx (hatched columns) cells in the absence (−) or presence (+) of Pgp siRNA (siR Pgp). When indicated, cells were transfected with the wild-type Pgp gene (wt Pgp) or with the siRNA-resistant Pgp gene (mu Pgp). Cells were incubated with 5 μmol/L doxorubicin for 6 h, and then doxorubicin accumulation was measured as described in Materials and Methods. Measurements were done in duplicate. Columns, mean (n = 3); bars, SE. *, P < 0.01 versus HT29; **, P < 0.001 versus HT29; †, P < 0.01 versus HT29-dx; ‡, P < 0.001 versus HT29-dx; ◊, P < 0.001 versus the respective condition without Pgp siRNA. B. HT29 and HT29-dx cells were treated as indicated in A and then lysed and subjected to Western blot analysis of Pgp (see Materials and Methods for details). The anti-GAPDH antibody was used as a control of equal protein loading (see Materials and Methods for details). The figure is representative of three experiments with similar results. C. HT29 (white columns) and HT29-dx (hatched columns) cells were cultured in the absence (CTRL) or presence of siRNA (siR) of RhoA and Pgp, alone or together. Silencing of the target proteins was done as indicated in Materials and Methods, and then cells were incubated for 6 h with 5 μmol/L doxorubicin and checked for intracellular drug content. Measurements were done in duplicate. Columns, mean (n = 3); bars, SE. *, P < 0.01 versus HT29; †, P < 0.01 versus HT29-dx; ‡, P < 0.001 versus HT29-dx; ◊, P < 0.001 versus Pgp siRNA.
subjected to overnight immunoprecipitation using a rabbit polyclonal anti-nitrotyrosine antibody (diluted 1:1,000; Upstate). Proteins were resolved by SDS-PAGE, transferred to PVDF membrane sheets, and probed with goat polyclonal antibodies (0.1 μg/mL) for 90 min at 4°C. Western blotting experiments were performed with enhanced chemiluminescence (Immun-Star, Bio-Rad Laboratories). The membrane was washed again with PBS-Tween and proteins were detected by enhanced chemiluminescence (Immun-Star, Bio-Rad Laboratories). The equal loading of proteins was checked by measuring the level of GAPDH, used as the product of a housekeeping gene.

IKK Activity Assay

IKK activity was measured as described previously (6). Cells were washed with ice-cold PBS and solubilized in 0.5 mL of lysis buffer. To purify IKK complex, equal amounts of the whole lysate (0.5 mg cell proteins per test) were immunoprecipitated with an anti-IKKα antibody (from rabbit, diluted 1:200 in PBS-1% BSA) for 90 min at 4°C. Immunoprecipitated proteins (0.1 μg) were incubated with 1 mmol/L ATP in the presence of the proteasome inhibitor MG132 (10 μmol/L). To provide the reaction mix with IκBα protein, the substrate of IKK, 30 μg of total cellular lysate of SV40-positive HMM cells, obtained under nondenaturing conditions, were added. SV40-positive HMM cells were chosen because they were previously shown to exhibit high basal levels of IκBα (39). MG132 was added during the assay to avoid the phosphorylated IκBα protein being destroyed by proteasome possibly still present in the cell lysates. The reaction was carried out at 30°C for 30 min and stopped with 30 μL of Laemmlli buffer. Finally, the samples were subjected to electrophoresis in a 12% SDS-PAGE, transferred to PVDF membrane sheets, and probed with an anti-IκBα antibody (from rabbit, diluted 1:250 in PBS-1% BSA) and an anti-phospho(Ser22)-IκBα antibody (from mouse, diluted 1:250 in PBS-1% BSA), respectively.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation assay was done following the recommendations of the manufacturer (Upstate) with some modifications. Briefly, cells were plated in 60-mm-diameter dishes (400,000 per dish) and transfected with RhoA siRNA (as described above); after 72 h, they were incubated with formaldehyde (final concentration, 1%) for 10 min at 37°C to cross-link proteins to DNA. The cross-linking reaction was quenched by the addition of one-tenth volume of 1.25 mol/L glycine, giving 125 mmol/L final concentration. Cells were washed twice with ice-cold PBS, resuspended in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 5 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0)] containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL peptatin A, and kept on ice for 30 min. Then, cell lysates were sonicated on ice with a Hielscher UP200S ultrasound sonicator (3 × 40 s, amplitude 40%, cycle 1; Hielscher Ultrasonics GmbH) until cross-linked chromatin was sheared to yield DNA fragments between 200 and 1,000 bp. One tenth of whole lysate was used to quantitate the amount of DNA present in different samples and considered as “total input DNA.” Supernatants were incubated with salmon sperm DNA/protein agarose-50% slurry to reduce nonspecific background. Immunoprecipitation was then done overnight at 4°C with 5 μg of anti-NF-κB p65 (Upstate) or without antibody (negative control). These supernatants were supplemented with 5 mol/L NaCl and heated overnight at 65°C to revert protein-DNA cross-links. The immunocomplexes were further treated with DNase- and RNase-free proteinase K, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. PCR was done with specific primers corresponding to a sequence within the promoter region of the human iNOS gene (p1 primer: 5′-GAGGGCGTCTCCCA- GAACCAAG-3′; p2 primer: GCTGGGCTACTGACCAG- CAGTTCCG-3′), as reported (40).

Measurement of NOS Activity

Cells grown at confluence in 35-mm-diameter Petri dishes, after incubation under the experimental conditions described in Results, were detached by trypsin/EDTA, washed with PBS, resuspended in 0.3 mL of HEPES/EDTA/DTT buffer [20 mmol/L HEPES, 0.5 mmol/L EDTA, 1 mmol/L DTT (pH 7.2)], and sonicated on crushed ice with two 10-s bursts. NOS activity was measured on 100 μg of cell lysates with the Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase kit (Oxford Biomedical Research). The results were expressed as nmol nitrite/min/mg cell proteins.

Doxorubicin Accumulation

The intracellular accumulation of doxorubicin was measured as described elsewhere (9) using a Perkin-Elmer LS-5 spectrophotometer. Excitation and emission wavelengths were 475 and 553 nm, respectively. A blank was prepared in the absence of cells in each set of experiments and its fluorescence

FIGURE 11. Schematic representation showing the sequence of steps by which RhoA siRNA decreases the efflux of doxorubicin and enhances the drug cytotoxicity via NF-κB activation, NOS activation, MRP3 nitration, and Pgp down-regulation.
was subtracted from that measured in each sample. Fluorescence was converted in ng doxorubicin/mg cell proteins using a calibration curve prepared previously.

**Extracellular LDH Activity**

After a 6-h incubation under different experimental conditions in the presence of 5 μmol/L doxorubicin, LDH activity was measured in the extracellular medium and in the cell lysate, as previously described (6, 41), as an index of the cytotoxic effect of doxorubicin. Absorbance at 340 nm was measured for 10 min with a Lambda 3 spectrophotometer (Perkin-Elmer). The reaction kinetics was linear throughout the time of measurement. Both intracellular and extracellular enzyme activity was expressed as μmol NADH oxidized/min/dish, and then extracellular LDH activity was calculated as percentage of the total LDH activity in the dish. In selected experiments, cells were pretreated with packed human erythrocytes (10 μL/mL of culture medium), the NOS inhibitor L-NMMA (2 mmol/L), or a specific NFκB nuclear translocation inhibitor (SN50, 25 μmol/L; Calbiochem), added 1 h before the 6-h incubation period with doxorubicin.

**Real-time PCR**

Total RNA was obtained as previously described (42). Total RNA (5 μg) was resuspended in 50 μL of PCR buffer [200 mmol/L Tris-HCl (pH 8.4), 500 mmol/L KCl, 5 mmol/L MgCl₂, 100 mmol/L DTT, 5 mmol/L deoxynucleotide triphosphate, 250 ng random primers] and retrotranscribed by 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in the presence of 40 μL RNAseOUT recombinant RNase inhibitor (Invitrogen). Real-time reverse transcription-PCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s instructions. Sequence-specific oligonucleotide primers (purchased from Sigma Chemical) were the following: human Pgp, 5'-TGCTGGACGGGTCTCACG-3' (forward) and 5'-ATAGCGAATCTGTCGACAG-3' (reverse); human iNOS, 5'-ACAACAAAATTCAGGTACGCTGTG-3' (forward) and 5'-TCTGATGAAATCTGACGAAAGG-3' (reverse); and human GAPDH, 5'-GAAAGGTAAGGTCCGAGTGTG-3' (forward) and 5'-CATGGTGATCATATTTGGAAG-3' (reverse). Thermal cycling conditions were the following: activation of iTaq DNA polymerase at 95°C for 3 min followed by 45 cycles of amplification at 94°C for 30 s, 55°C (Pgp) or 58°C (iNOS and GAPDH) for 30 s, and 72°C for 30 s. To detect the log phase of amplification, the fluorescence level (quantification of product) was determined at each cycle. The cycle at which the fluorescence reached the threshold was recorded, averaged between triplicates, and normalized to the averaged cycle of threshold value for GAPDH. Fold change in expression with respect to control (HT29 cells) was calculated for all samples using the Bio-Rad Laboratories Software Gene Expression Quantitation.

\( V_{\text{max}} \) of Doxorubicin Efflux

To measure the maximal doxorubicin efflux through ABC transporters, we incubated cells (150,000, plated in 35-mm-diameter Petri dishes) with 250 μmol/L doxorubicin for 10 min; under these experimental conditions, ABC transporters work at their \( V_{\text{max}} \) in HT29 and HT29-dx cells (9). In a set of dishes (set B), cells were washed with PBS, resuspended in 1 mL of ethanol/0.3 N HCl, and analyzed for the intracellular drug content as described above. In parallel, in a second set of dishes (set A), the cells, after incubation with doxorubicin under the same experimental conditions, were washed with PBS, left for 10 min further in PBS at 37° C, and then washed again, resuspended in ethanol/HCl, and tested for intracellular doxorubicin. Aliquots of cells suspensions (50 μL) were used to measure the intracellular protein content by bicinchoninic acid kit. The difference of doxorubicin concentration between B and A samples under each experimental condition was expressed as ng of doxorubicin extruded/min/mg of cell proteins (dc/dt).

**ATPase Activity Assay**

Cells were lysed in buffer A [50 mmol/L HEPES, 750 mmol/L KCl, 200 mmol/L sucrose, 10 mmol/L NaHCO₃ (pH 7.4)], supplemented with protease inhibitor cocktail set III (Calbiochem), and centrifuged at 13,000 × g for 5 min. Supernatant was collected and centrifuged again at 100,000 × g for 1 h at 4°C, and then the pellet was resuspended in 1 mL of buffer B [20 mmol/L HEPES, 160 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 0.5% Triton X-100 (pH 7.4)]; 100 μg of proteins were immunoprecipitated overnight with the rabbit polyclonal anti-Pgp antibody (diluted 1:100; Santa Cruz Biotechnology) or with the goat polyclonal anti-MRP3 antibody (diluted 1:100). Samples were washed twice with 1 mL of buffer B, supplemented with 2 mmol/L DTT, and then subjected to the following investigations. Immunoprecipitated proteins (10 μg) were directly probed with the same anti-Pgp or anti-MRP3 antibodies (diluted 1:250 in PBS-1% BSA) by Western blot analysis to measure total Pgp and MRP3 protein (data not shown). Fifty micrograms were mixed with 2 mmol/L ATP, 2.5 mmol/L phosphoenolpyruvate, 7.5 units pyruvate kinase, and 8.0 units LDH to check ATPase activity, as previously described (43). The reaction was started by adding 0.25 mmol/L NADH and was followed for 10 min, measuring the absorbance at 340 nm with a Lambda 3 spectrophotometer (Perkin-Elmer). The reaction kinetics was linear throughout the time of measurement. The NADH oxidation rate (expressed as μmol NADH oxidized/min/mg cell proteins) of each sample was subtracted from the one obtained in the absence of Pgp/MRP3. The ATP hydrolysis rate was calculated stoichiometrically and ATPase activity was expressed as μmol ATP hydrolyzed/min/mg cell proteins.

**Immunofluorescence Studies**

Cells (50,000 per well) were plated in eight-well Permanox slides in RPMI 1640 plus 10% FBS the day before the immunofluorescence analysis. Nonpermeabilized cells were fixed in 3.5% paraformaldehyde containing 2% sucrose for 15 min at room temperature and washed in PBS. Cells were then incubated for 60 min at room temperature with anti-Pgp monoclonal antibody (500 μg/mL) followed by incubation for 30 min at room temperature with the appropriate
FITC-conjugated secondary antibody (DAKO). The slides were then mounted with Vectashield mounting medium (Vector Laboratories, Inc.) and examined. Control experiments included the incubation of cells with nonimmune isotypic control antibodies followed by the appropriate labeled secondary antibodies. Pgp expression was analyzed semiquantitatively by measuring fluorescence intensity by digital image analysis (Windows MicroImage, version 3.4; CASTI Imaging) on images obtained using a low light video camera (Leica DC100) with a 180-μm-diameter field. The results were expressed as relative fluorescence intensity on a scale from 0 (fluorescence of background of tissue) to 255 (fluorescence of standard filter). For each experimental point, a minimum of five microscopic fields was examined.

DNA Constructs and Mutagenesis

The vector pHa containing the Pgp (MDR1) complete cDNA was purchased from Addgene. For mutagenesis, Pgp was subcloned into pCDNA3 (Invitrogen). The pHa-Pgp construct was digested with SpeI (Fermentas International, Inc.), blunted with Klenow (Roche), and then digested with XhoI (Fermentas International). The SacII-blunt-XhoI fragment was cloned into the EcoRV/XhoI sites of pCDNA3. Pgp mutant construct resistant to specific siRNA was then generated by PCR-based mutagenesis (Stratagene). The primers used for mutagenesis corresponded to siRNA sequence (Santa Cruz Biotechnology) and carried three mutations. Mutations were confirmed by DNA sequencing (data not shown).

Pgp Transfection

HT29 and HT29-dx cells were transfected either with the Pgp wild-type construct or with the Pgp mutated construct, alone or together with the Pgp siRNA. Cells (200,000 per dish) were plated in 35-mm-diameter dishes and cultured in RPMI 1640 containing 10% FBS. After 24 h, cells were washed once and incubated for 6 h with 1 mL siRNA transfection medium, containing 5 μL of siRNA transfection reagent, in the presence or absence of 50 pmol of Pgp siRNA. At the end of the incubation, 1 mL of RPMI 1640, containing 6 μL of jetPEI transfection reagent (Polyplus-transfection SA BIOPARC), 3 μg DNA (Pgp wild-type construct or Pgp mutated construct), 1% penicillin/streptomycin, and 20% FBS, was added. After an overnight incubation, cells were washed and cultured for 72 h in RPMI 1640 with 1% penicillin/streptomycin and 10% FBS. To assess the Pgp transfection and the siRNA effectiveness, the expression of Pgp was checked by Western blot analysis, as reported above. Cell viability was not affected by transfection because the LDH release in the supernatant after 24, 48, and 72 h from the transfection was the same as in nontransfected cells (data not shown).

Statistical Analysis

All data in text and figures are provided as mean ± SE. The results were analyzed by a one-way ANOVA and Tukey’s test. P < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest exist.


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